Focal adhesion kinase coordinates costamere-related JNK signaling with muscle fiber transformation after Achilles tenotomy and tendon reconstruction

Ferrié, Céline ; Kasper, Stephanie ; Wanivenhaus, Florian ; Flück, Martin

Abstract: Achilles tendon rupture necessitates rapid tendon reattachment to reinstate plantar flexion before affected muscles deteriorate through muscle fiber atrophy and transformation. The implicated process may involve alterations in sarcolemmal sites of myofibril attachment (costameres), which control myofibrillogenesis via a mechano-regulated mechanism through integrin-associated focal adhesion kinase (FAK). We assessed the contribution of FAK to alterations in fiber type composition and expression of costamere-associated structural proteins, the phosphorylation status of Y397-FAK and downstream mTOR/JNK-P70S6K hypertrophy signaling in rat soleus muscle after Achilles tenotomy and tendon repair. Achilles tenotomy induced a profound deterioration of muscle composition 14 days, but not 4 days, following tendon release, comprising specifically increased area percentages of fast type fibers, fibers with internal nuclei, and connective tissue. Concomitantly, expression of costameric proteins FAK and meta-vinculin, and phosphorylation of T421/S424-P70S6K and T183/Y185-JNK was elevated, all of which was mitigated by tendon reattachment immediately after release. Overexpression of FAK in soleus muscle fibers and reattachment corrected the expression of meta- and gamma-vinculin isoforms to the lower levels in mock controls while further enhancing T183/Y185-JNK phosphorylation and levels of FAK C-terminus-related inhibitory proteins. Co-overexpression of the FAK inhibitor, FRNK, lowered FAK-overexpression driven Y397-FAK phosphorylation and T183/Y185-JNK phosphorylation. FAK levels correlated to molecular and cellular hallmarks of fiber degeneration. The findings demarcate the window between 4 and 14 days after tenotomy as costamere-dependent muscle transformation process, and expose that FAK overexpression prevents molecular aspects of the pathology which within the study limitations does not result in the mitigation of muscle fiber degeneration.250 words.

DOI: https://doi.org/10.1016/j.yexmp.2019.03.006

Ferrié, Céline; Kasper, Stephanie; Wanivenhaus, Florian; Flück, Martin (2019). Focal adhesion kinase coordinates costamere-related JNK signaling with muscle fiber transformation after Achilles tenotomy and tendon reconstruction. Experimental and Molecular Pathology, 108:42-56.
DOI: https://doi.org/10.1016/j.yexmp.2019.03.006

Experimental and Molecular Pathology 108 (2019) 42–56

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Focal adhesion kinase coordinates costamere-related JNK signaling with muscle fiber transformation after Achilles tenotomy and tendon reconstruction

Céline Ferrié[a], Stephanie Kasper[a], Florian Wanivenhaus[b], Martin Flück[a,*]

[a] Laboratory for Muscle Plasticity, Department of Orthopedics, University of Zurich, Balgrist Campus, Zurich, Switzerland
[b] Department of Orthopedic Surgery, Balgrist University Hospital, Zurich, Switzerland

ARTICLE INFO

Keywords:
Tenotomy
Gene therapy
Costamere
FAK
Vinculin
mTOR/P70S6K pathway

ABSTRACT

Achilles tendon rupture necessitates rapid tendon reattachment to reinstate plantar flexion before affected muscles deteriorate through muscle fiber atrophy and transformation. The implicated process may involve alterations in sarcolemmal sites of myofibril attachment (costameres), which control myofibrillogenesis via a mechano-regulated mechanism through integrin-associated focal adhesion kinase (FAK).

We assessed the contribution of FAK to alterations in fiber type composition and expression of costamere-associated structural proteins, the phosphorylation status of Y397-FAK and downstream mTOR/JNK-P70S6K hypertrophy signaling in rat *soleus* muscle after Achilles tenotomy and tendon repair.

Achilles tenotomy induced a profound deterioration of muscle composition 14 days, but not 4 days, following tendon release, comprising specifically increased area percentages of fast type fibers, fibers with internal nuclei, and connective tissue. Concomitantly, expression of costameric proteins FAK and meta-vinculin, and phosphorylation of T421/S424-P70S6K and T183/Y185-JNK was elevated, all of which was mitigated by tendon reattachment immediately after release. Overexpression of FAK in *soleus* muscle fibers and reattachment corrected the expression of meta- and gamma-vinculin isoforms to the lower levels in mock controls while further enhancing T183/Y185-JNK phosphorylation and levels of FAK C-terminus-related inhibitory proteins. Co-overexpression of the FAK inhibitor, FRNK, lowered FAK-overexpression driven Y397-FAK phosphorylation and T183/Y185-JNK phosphorylation. FAK levels correlated to molecular and cellular hallmarks of fiber degeneration. The findings demarcate the window between 4 and 14 days after tenotomy as costamere-dependent muscle transformation process, and expose that FAK overexpression prevents molecular aspects of the pathology which within the study limitations does not result in the mitigation of muscle fiber degeneration.250 words.

1. Introduction

Release of the Achilles tendon (tenotomy) is a relatively frequent clinical situation occurring in consequence of a traumatic incidence or therapeutic intervention to correct an equinus deformity (Egger and Berkowitz, 2017; Ramanujam and Zgonis, 2017). Achilles tenotomy produces considerable pain and renders motor tasks involving the plantar flexor muscles, *M. plantaris*, gastrocnemius and soleus, hard to impossible. Recovery of severed (Achilles) tendons is best when tendons are reattached early after tenotomy although the connectivity of the severed tendon stump to the calcaneus bone recovers spontaneously (Jakubiec-Puka et al., 1992; Abrams et al., 2000). Incomplete functional restoration of plantar flexion, as assessed through passive and active tension, appears to be related, although not being explained by the weakening and shortening of the involved plantar flexor/ankle extensor muscle-tendon composite through degenerative alterations of the injured tendon and muscle fiber (Abrams et al., 2000; Jamali et al., 2000).

Abbreviations: AP-1, Activator protein 1 (heterodimer of jun/fos transcription factors); c-JUN, Transcription factor (seventeenths clone of the cellular isoform of the avian sarcoma virus); FAK, Focal adhesion kinase; FRNK, FAK-related non-kinase; JNK, c-JUN N-terminal kinase; mTOR, Mammalian target of rapamycin; P70S6K, Phosphoprotein 70 ribosomal protein S6; p65FAK, FAK C-terminus related protein at 65 kDa; p55FAK, FAK C-terminus related protein at 55 kDa; pS63-cJUN, Serine 63 phosphorylated c-JUN; pS2448-mTOR, Serine 2448 phosphorylated mTOR; pT421/S424-P70S6K, Threonine 421 and serine 424 phosphorylated P70S6K; pT183/Y185-JNK, Threonine 183 and tyrosine 185 phosphorylated JNK; FAK, Focal adhesion kinase; Y397-FAK, Tyrosine 397 of FAK

* Corresponding author.
E-mail address: mflueck@research.balgrist.ch (M. Flück).

https://doi.org/10.1016/j.yexmp.2019.03.006
Received 25 June 2018; Received in revised form 29 January 2019; Accepted 12 March 2019
Available online 15 March 2019

The cellular processes underlying extensor muscle degeneration after tenotomy, and Achilles tenotomy in particular, involve a loss in contractile material (i.e. sarcomeres), reducing the length and mean cross sectional area of muscle fibers, a slow-to-fast transformation of the muscle phenotype, and a concomitant increase in connective tissue per muscle tissue (Sunderland and Lavarack, 1959; Baker and Hall-Craggs, 1980; Jakubiec-Puka et al., 1992; Jamali et al., 2000). Both the removal of sarcomeres during the atrophic process, and the exchange of slow with fast type sarcomeres during muscle fiber transformation, involve modifications in focal sites of adhesion in the sarcolemma (so called costameres), which hold sarcomeres in register (Goll et al., 2008; Klossner et al., 2013; Li et al., 2013; Jaka et al., 2015). Costameres revolve as an assembly of cytoskeletal and signaling molecules around two major receptors for the extracellular matrix protein laminin, the dystrophin-glycoprotein complex and the integrin-vinculin-talin complex (Sparrow and Schock, 2009; Peter et al., 2011). Costameres therefore mediate connections between myofibrils on the fiber interior and the extracellular matrix on the fiber outside (Pardo et al., 1983) and provide a substrate for the assembly of myofibrils (Sparrow and Schock, 2009; Garcia-Pelagio et al., 2011). Consequently, the cleavage of costameric proteins, as it occurs after tenotomy, and post mortem is understood to release myofibrils for proteolytic degradation (Bullard et al., 1990; Goll et al., 2003; McNeil and Kirchhausen, 2005; Goll et al., 2008; Ruoss et al., 2018b). Conversely, expressional up-regulation of costamere components, such as observed in anti-gravity muscle for dystrophin, dystroglycan, sarcoglycan, talin, meta- and gamma-vinculin, with weeks of reduced or increased load-bearing activity is associated with the transformation of myosin heavy chain composition and alterations in the cross-sectional area of muscle fibers (Sharp et al., 1997; Fluck et al., 2002; Chopard et al., 2005; Hoshijima, 2006; Klossner et al., 2013; Li et al., 2013; Ruoss et al., 2018b). Linear relationships between costamere component expression and anatomical estimates of muscle size such as the mean cross sectional area of muscle fibers and the pennation angle of muscle fiber bundles (i.e. fascicles) highlight the interdependence between costamere component expression and muscle anatomy (Narici et al., 2011; Li et al., 2013; Fluck et al., 2014).

The integrin-associated protein tyrosine kinase FAK plays a critical role for the assembly (costamerogenesis) and turnover of costameres and the reorganisation of sarcomeres via its role as a phosphotransferase and scaffold for the recruited focal adhesion proteins (Chu et al., 2011; Santos et al., 2011; Graham et al., 2015). FAK is part of the load- and fiber type-related mechanism of fully-developed muscle tissue that controls the expression of costamere components (meta-vinculin and b1-integrin), governs the slow oxidative gene expression program, and regulates signaling molecules that control myofibrillar protein synthesis (reviewed in (Oktay et al., 1999; Durieux et al., 2009; Klossner, Durieux et al., 2009; Klossner et al., 2013; Graham et al., 2015)). The biological activity of FAK is regulated by load-bearing contractile activity through an acute increase in the phosphorylation of tyrosine residue 397 on FAK (Y397-FAK), and auxiliary sites such as Y576 and Y577 (Klossner et al., 2009; Rahnert and Burkholder, 2013; Graham et al., 2015); and this includes alterations in FAK content with chronic alterations in load-bearing (Gordon et al., 2001; Klossner et al., 2013; Graham et al., 2015). Mechano-regulation downstream of FAK includes the phosphorylation of amino acids T421/S424 on P70S6K in skeletal muscle (Durieux et al., 2009; Klossner et al., 2009); and S2448 phosphorylation of mTOR (Zanchi and Lancha Jr., 2008; Graham et al., 2015) and T183/Y185 phosphorylation of JNK (Oktay et al., 1999; Zanchi and Lancha Jr., 2008; Graham et al., 2015); all of which lead to P70S6K-mediated activation of protein-synthesis (Fujii et al., 2004; Zanchi and Lancha Jr., 2008; Martin et al., 2014; Graham et al., 2015). Additionally, elevated JNK phosphorylation may increase N-terminal phosphorylation of the transcription factor c-JUN, which controls the promoter activity of genes being associated with muscle differentiation (Dawes et al., 1996; Paradis et al., 1996; Herdegen et al., 1998; Andreucci et al., 2002).

Y397 phosphorylation and localisation of FAK to focal adhesions is further inhibited by an endogenous process which involves protein products that stem from its C-terminus (Zak et al., 2017). For instance, FRNK, which corresponds to the C-terminal focal adhesion targeting domain of FAK is expressed in a muscle fiber type-related way and interferes with Y397 phosphorylation of FAK and load-regulated and costamere-related expression of the slow oxidative gene program in soleus muscle (Fluck et al., 2002; Durieux et al., 2009; Klossner et al., 2013; Zak et al., 2017). As well, in numerous cell types, catabolic situations which lead to cell death, have similarly as seen for the costameric proteins vinculin and talin (Goll et al., 2003; Goll et al., 2008; Ruoss et al., 2018a, 2018b), been found to give rise to proteolytic fragments from the C-terminus of FAK which inhibit the FAK phosphorylation and localisation of FAK to focal adhesions (Carragher et al., 2001; Zak et al., 2017).

The aim of this investigation was to provide information on the quantitative, temporal involvement of costamere-dependent mechanisms to muscle fiber degeneration after Achilles tendon rupture and repair. The hypothesis was that the expression levels of FAK-modulated costamere components (FAK, FRNK, meta- and gamma-vinculin) in rat soleus muscle are modified after Achilles tenotomy (Klossner, Li et al., 2013) along with a fall in the activation status of costamere-associated protein synthetic signaling factors (FAK, mTOR, P70S6K, JNK), and that these molecular alterations would be reflected in altered linear relationship to structural hallmarks of muscle fiber degeneration (such as central core fibers, slow-to-fast fiber transformation, reductions in mean cross sectional area of muscle fibers (Jamali et al., 2000). We further assumed that a certain degree of muscle fiber degeneration would occur even when the severed tendon is immediately reattached, but that concurrent somatic transfection with an expression plasmid for FAK would mitigate aspects of the degenerative process due to an enhanced drive for expression of costamere components and protein synthetic signaling. The specificity of FAK signaling due to FAK overexpression was tested in experiments co-overexpressing the FAK inhibitor FRNK.

2. Methods

2.1. Study design

Three experimental interventions were performed with rats (Fig. 1). As surgical procedures that target muscle tissue produce important compensatory effects in agonistic muscle groups (reviewed in (Lu et al., 1999)), the specificity of effects was controlled by comparing assessed parameters to mock manipulations in the contralateral controls, and without somatic transfection. The first experiment comprised a time course experiment to study the effect of unilateral Achilles tenotomy of the right *soleus* muscle tendon compared to the mock-treated *soleus* tendon in the contralateral leg (termed 'tenotomy groups'). A time course spanning two weeks after tenotomy was chosen because this duration produces pronounced muscle degeneration in the studied muscle (Baker and Hall-Craggs, 1980; Jakubiec-Puka et al., 1992; Jamali et al., 2000), including samples from earlier time points when first effects on FAK-associated signaling molecules (Aronson et al., 1997; Midwood and Schwarzbauer, 2002; Klossner, Li et al., 2013) were reported to occur in rat *soleus* muscle with Achilles tenotomy and muscle unloading. The second experiment consisted of an intervention in which *soleus* muscles were transfected with expression plasmid, pCMV-FAK, its empty control (pCMV), or not-transfected, before transection and immediate reconstruction via re-attachment of the right *soleus* tendon was performed (termed 'reattachment groups'). In a third experiment, left and right *soleus* muscles, respectively, which were not tenotomised, were transfected with empty control (pCMV) or expression plasmid for FRNK (pCMV-FRNK), additional to pCMV-FAK to identify the effect of FAK inhibition on signaling.

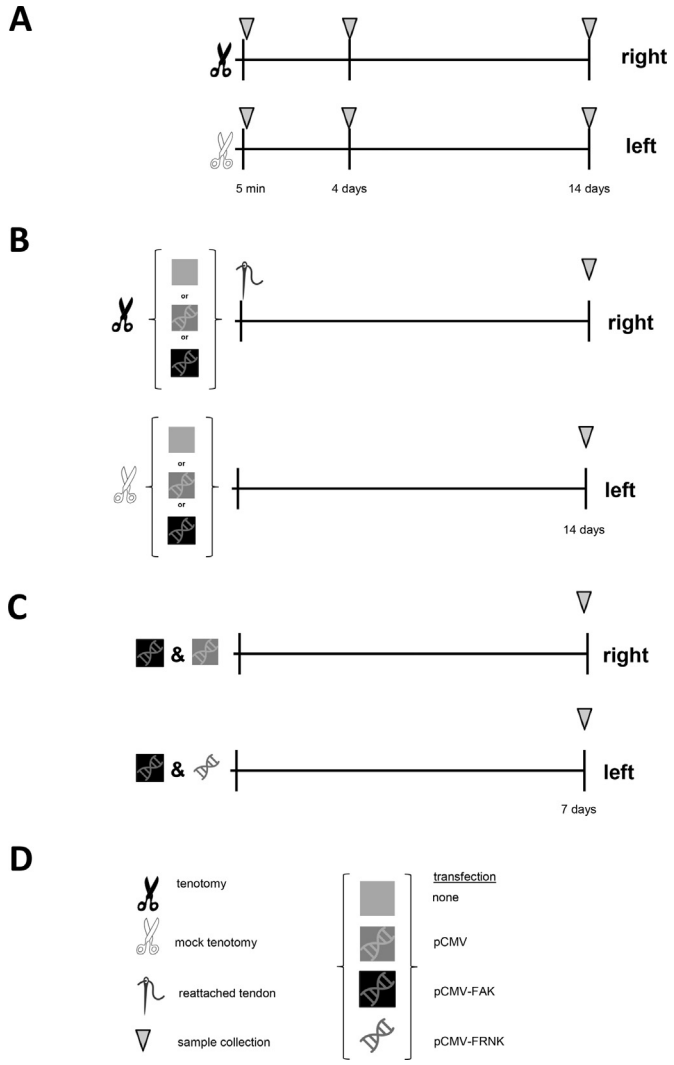

Fig. 1. Experimental design. Scheme of the three experimental interventions to study the temporal response of *soleus* muscles 5 min, 4 days or 14 days after Achilles tenotomy and cage activity (A, tenotomy groups), to assess the response to 14 days after Achilles tenotomy and reattachment, in function of transfection with the indicated plasmids compared to mock controls (B, reattachment groups), and assess the specificity of FAK signaling (C, FRNK co-overexpression). D) Used symbolism.

The first experiment was performed in 3 series, whereby the animals were randomly assigned to groups of six animals, which were subjected to tenotomy and thereafter allowed to recover for a same given duration, i.e. 5 min, 4 days or 14 days before the *soleus* and *gastrocnemius medialis* muscles were collected under anesthesia from the tenotomized and mock-treated leg. For the 4 day and 14 day time point, the wound was closed, and animals allowed moving freely before muscles were collected. The second experiment was run in 3 series. In the first two series rats, right and left *soleus* muscles were surgically exposed and transfected with pCMV-FAK or pCMV plasmid, immediately before the right *soleus* tendon was transected and reattached; and the left *soleus* tendon was mock-transected. In the third series animals, *soleus* muscles were exposed, but not transfected and the right *soleus* tendon was transected and reattached. The left *soleus* tendon was mock transected. *Soleus* and *gastrocnemius medialis* muscles were harvested 14 days after tendon reattachment. The third experiment was run in one series for which animals were not subjected to tenotomy, and the soleus muscles were harvested 7 days after tendon release. The first and second experiments were run at the University of Zurich. The third experiment was conducted at the University of Berne.

Cryosections were prepared from the belly portion of the collected muscles, and subjected to histological analysis of muscle composition, and pooled for biochemical analysis of protein expression by immunoblotting and electrochemiluminescence-based immunoassay from muscle homogenate.

2.2. Animal care

The experimental procedures were approved by the animal protection commission and ethics commission of the Canton of Zurich, Switzerland (first and second experiment); and the Canton of Berne, Switzerland (third experiment), respectively. The experiments were conducted in animal facilities that were approved by the Institutional Animal Care and Use Committees of the respective University. Twelve-week old Wistar female pathogen-free rats (Janvier Labs, FRANCE for the first and second experiment; and Charles River Laboratory, L'Arbresles, FRANCE for the third experiment) were housed in a temperature and humidity-controlled facility. Upon arrival and before the surgical procedure (start), animals were acclimatized for 1 week in new cages in groups of 3 animals with food and water ad libitum, and 2 animals per cage after surgery. The healthiness and the stress level of each rat were monitored daily over the entire duration of the experiment with a score table.

To reduce pain, doses of analgesic (Buprenorphin 50 µg/kg body weight (Temgesic, Reckitt Benckiser AG, Switzerland)) were administered subcutaneously half an hour before the operation took place and five minutes before inhalation anesthesia was terminated. Further doses of analgesic were administered during a 24 h-follow-up according to the postoperative pain monitoring.

2.3. Tenotomy

These experiments were performed essentially as described before (Klossner et al., 2013). Rats were anesthetized with 2–4% isoflurane (Rothacher-Medical GmbH, Switzerland, #ISO250ml) and oxygen (1 L min^{-1}). Anesthesia was maintained in 3% isoflurane during the entire surgical procedure with the help of a commercial tube system (Provet, Lyssach b. Burgdorf, Switzerland).

On the right leg of each animal, an approximately 4-mm-long incision was made at the dorsal side of the lower leg towards the tendinous end of the *gastrocnemius* muscle taking care underlying blood vessels and nerves were not damaged. The Achilles tendon was exposed by lifting the *soleus* portion of the Achilles tendon with a pair of forceps and the tendon was transected with a scalpel. Subsequently the skin is closed with a Novosyn® thread (5–0, DS16, Braun Medical AG, Switzerland). Left muscles did undergo a mock tenotomy in which the *soleus* muscle tendon was exposed, but not dissected. Subsequently, animals were released from the anesthesia and allowed to gain consciousness under normal oxygenation in a cage.

2.4. Tenotomy and tendon reattachment

A skin incision was performed longitudinally on the posterolateral aspect of the right ankle. The *soleus* part of the Achilles tendon was exposed and modified Mason-Allen stitches were placed, using Silkam® non-absorbable thread (4–0, DS16, B.Braun Medical AG, Switzerland), on either side of the future cut. The *soleus* Achilles tendon was cut between the stitches and immediately reattached by pulling the suture thread. Subsequently the skin was closed using a Novosyn® 5–0 thread. On the left side, serving as mock control for the reattachment, the *soleus* part of the Achilles tendon was treated as described for 'mock tenotomy'; i.e. it was surgically exposed with blunt forceps but the tendon was not dissected.

2.5. Muscle transfection

For the second experiment right and left *m. solei* were surgically exposed by a lateral split of the connective tissue sheet between the *gastrocnemius* and *tibialis anterior* muscles. Endotoxin-free empty plasmid (pCMV) or plasmid coding for FAK protein (pCMV-FAK), was injected at a concentration of 35 µg in 35 µL in a 0.9% NaCl solution into the deep and superficial region of the belly portion of the *soleus* muscle. Three trains of 80 × 100 µs pulses, each at 100 mA, were delivered using needle electrodes with a GET42generator (E.I.P.Electronique et Informatique du Pilat, Jonzieux, France). The third transfection experiment was carried out in an analogous manner but 25 µg of two plasmids each, i.e. pCMV and pCMV-FAK or pCMV-FRNK and pCMV-FAK, were injected in the right and left *soleus* muscle, respectively.

2.6. Muscle collection

At the end of the experiment, rats were anesthetized in 2–4% isoflurane and a circular incision was drawn around the heel just above the calcaneus followed by a long medial cut from the heel towards the knee. Subsequently, the musculature of the lower leg was exposed by stripping the skin towards the knee. The two tendons that insert distally into the lateral and medial head of the *gastrocnemius* muscle were clamped and the *soleus* muscle was exposed by reverting the triceps group over the popliteal fossa. The *soleus* muscle was grabbed with a pair of forceps and its proximal tendinous end was capped with a scalpel. The collected muscle was weighed in a microbalance before being frozen in liquid nitrogen-cooled isopentane (VWR International GmbH, Switzerland, #24872.298). The same procedure was carried out for the left *m. soleus*. All muscles were kept in sealed cryo-tube at −80 °C freezer until molecular analysis was performed.

2.7. Muscle sectioning

Cryosections were prepared in perpendicular direction to the major axes of *soleus* muscles with the help of a cryostat (CM3050, Leica, Germany). The ten first sections were laid on slides (Menzel, Superfrost) for future staining. The following one hundred sections were collected into two separate flat bottom tubes (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland), which were cooled in liquid nitrogen, and stored until further biochemical analysis (i.e. immunoblotting) at −80 °C.

2.8. Histological analysis of muscle fiber type composition and cross-sectional area

To quantify fast and slow fibers, two continuous sections of the right and left *soleus* were stained of each rat with antibodies against slow (Merck Millipore Corporation, Switzerland, #MAB1628) and fast type myosin heavy chain (Sigma-Aldrich Chemie GmbH, Switzerland, #M4276), respectively. Then, overlapping images were taken at a 10× magnification (Olympus IX50 microscope, USA) from the stained sections and assembled to reveal full coverage of the section. Image J software (version 1.46, National Institutes of Health, USA) was used to determine the number of fast, slow or hybrid fibers in each section. Cryosections of right and left *soleus* were processed using the Goldner trichrome method. Overlapping images were taken at a 10× magnification (Olympus IX50 microscope, USA), assembled and analyzed based on morphometric principles (Mayhew, 1991). In brief, a grid was superimposed and the images printed at a final 7700-fold magnification on DIN A3 paper. The number of muscle fiber types, and fibers with internal nuclei or central cores was estimated in every fourth square under application of the forbidden line rule. Subsequently the squares were subdivided into 16 equal sized squares and the mean cross-sectional area (MCSA) of fibers was estimated by point counting of the fiber profiles. On average 299 fibers were evaluated per *soleus* muscle.

2.9. Homogenate preparation

Frozen sections were homogenized in 200 µL of RIPA buffer (2% Triton X-100, 1% NP-40, 300 mM NaCl, 20 mM Tris base, 2 mM EDTA, 2 mM EGTA) with a Polytron® (PT 1200 E, Kinematica AG, Switzerland). Total protein content was determined with the Pierce® BCA protein assay kit (Thermo Fischer Scientific, USA).

2.10. Immunoblotting

10 µg protein of each homogenates, was analyzed for the content of FAK, FRNK, gamma- and meta-vinculin by SDS-PAGE, subsequent immunoblotting and enhanced chemoluninescence-based signal detection essentially as described using established antibodies (Klossner et al., 2013; Franchi et al., 2018; Ruoss et al., 2018b). The dilutions of the first antibodies were 1:1000 for the polyconal antiserum LuLu to detect FAK and FRNK (Fluck et al., 1999), as well as the polyclonal antibody against vinculin to gamma- and meta-vinculin (Sigma-Aldrich Chemie GmbH, Switzerland, #V9264). The dilutions for the appropriate horseradish peroxidase-conjugated goat secondary antibodies against rabbit IgG (#55676, MP Biomedicals, Zurich, Switzerland) or against mouse IgG (#9917, Sigma, Buchs, Switzerland) were both 1:10000. Background corrected band intensities were related to the intensity of the actin band for the respective homogenate sample as identified on the Ponceau S stained membrane. Samples were loaded with a specific analytic scheme comprising the inclusion of a reference sample on each blot. For each immunoblot the actin-related values were related to the respective values from the reference samples and the resulting values from the different immunoblots were pooled.

2.11. Electrochemiluminescence-based immunoassay

Multiplex kits based on electro-chemiluminescence technology were used to quantify the phosphorylation of selected signaling factors through the measure of critical auto-regulatory sites. This comprised P70S6K (T421, S424), mTOR (S2448), JNK (T183, Y185), cJUN (S63) and respective total proteins in supernatants of RIPA-based homogenates of *soleus* muscle (K15114D-1, K15170D-1, K15111D-1, and K151CGD-2; Meso Scale Discovery, USA). Y397 phosphorylation of FAK and total FAK protein were measured using an U-PLEX development system (Meso Scale Discovery). An antibody directed against the N-terminal region of FAK (AF4467, R&D systems) and an antibody that detects the phosphorylation at tyrosine 397 (P00151, Boster Biological Technology) were biotinylated (Biotin-NH_2 labeling Kit, KT-221, Kamiya Biomedical Company) and used as capture antibodies. The C-terminal FAK antibody was purified from FAK serum “Lulu” (gift of Dr. Andrew Ziemiecki, University of Berne (Fluck et al., 1999)) using Ab-Pure Antibody Purification System (Innova Bioscience) and conjugated with SULFO-TAGTM (MSD GOLD SULFO-TAGTM NHS-Ester) to be used as detection antibody. The assay was validated against signals in samples of known FAK activity and amount (Durieux et al., 2009; Klossner et al., 2009; Klossner et al., 2013). Plates were read on a QuickPlex SQ120 Imager (Meso Scale Discovery, USA) as per the manufacturer's instructions. Homogenate corresponding to 33 µg of protein was loaded per well of the ELISA plate. The results of the ELISA were expressed in ECL counts and the values of phospho-protein were related to respective total protein expression of each kinase, that is, JNK, mTOR, P70S6K, FAK, to reveal the specific phosphorylation levels. As this was effectively a ratio of ECL counts, no units were assigned to phospho-protein levels.

2.12. Immunohistochemistry/fluorescence

Detection of FAK was carried out essentially as described by

incubation with specific antibodies (Durieux et al., 2009; Klossner et al., 2013). In brief, fixed and quenched cryosections were blocked in 3% bovine serum albumin-phosphate buffered saline and incubated with rabbit antibody A-17 (Santa Cruz, LabForce AG, Muttenz, Switzerland) directed against the N-terminus of FAK, then secondary anti-rabbit antibody being coupled to horse radish peroxidase with washing steps in between. Signal was developed histochemically, recorded with an Olympus IX50 microscope (Olympus, USA) and saved as tiff-file. Signal was verified in selected sections based in immunofluorescent detection deploying rabbit antibody A-17 (Santa Cruz) and an Alexa 488 coupled secondary antibody (Dako, Basel, Switzerland). Signal intensity was inspected visually on a projection of the tiff-files from the immunohistochemical staining on a computer screen. Fibers were assigned manually on an overlaid transparency as demonstrating enhanced or normal staining for FAK in the sarcoplasma or sarcolemma, and counted. The criteria for assigning an enhanced staining in the sarcoplasm to a given fiber, was that signal was identified throughout the sarcoplasm and was not only confined to 'dots' between the supposed myofibrils. Sarcolemmal staining was declared if more than half of the fiber periphery showed distinct positive FAK immunoreactivity. Immunohistochemical experiments were run in three series whereby two pCMV and two pCMV-FAK transfected sections were stained in one series. A minima of 100 fibers were counted per cross section.

2.13. Statistical analysis

Data were organized using MS-Excel (Microsoft, Kildare, Ireland) and exported into SPSS (version 23, IBM) to carry out statistical tests. A repeated measures ANOVA for the repeated factor leg (mock, tenotomy) and the factors reattachment (no, yes) and protein (FAK, FRNK, p65FAK, p55FAK) was performed to test the interaction effects of tenotomy and reattachment on the individual FAK-related proteins. The analysis of the other factors was run individually for each protein with repeated measures ANOVA. A repeated ANOVA for the repeated factor leg (mock, tenotomy) and the factor time (5 min, 4 days, 14 days) was performed to test the interaction effects of time x tenotomy. Subsequently, effects which met the criteria of significance (i.e. with p-values below 0.05), were localized post-hoc with a test for least significance difference. A repeated ANOVA for the repeated factor leg (mock, tenotomy) and the factor plasmid (no, pCMV, pCMV-FAK) was performed to test the interaction effects of plasmid x tenotomy-reattachment. The influence of pCMV-FRNK co-transfection on FAK-modulated phosphorylation of signaling factors was assessed with a repeated ANOVA for the factor plasmids (pCMV and pCMV-FAK; pCMV-FRNK and pCMV-FAK). Differences for effects demonstrating a p-value below 0.05 were localized post-hoc with a test for least significance difference. Linear relationships were calculated based on Pearson correlations; those relationships meeting $p < .05$ were imported into cytoscape (version 3.2.0; http://www.cytoscape.org/), displayed as organic hierarchical network and exported as png-file for figure assembly in MS-Office Powerpoint (Microsoft, Kildare, Ireland).

3. Results

3.1. Animals

Body mass data are shown in Table 1. At the start, one week before the surgical procedure, the animals of the reattachment group tended to be heavier than the animals being subjected to 14 days of tenotomy ($p = .07$). Body mass was increased by 7% over the duration of the intervention in the animals being subjected to 14 days of tenotomy. Body mass did not change during the intervention in the reattachment group ($p = .11$). The mass of the animals from the pCMV-FRNK co-transfection experiments increased by 8% during the intervention and was lower than the mass of the animals from the tenotomy, and reattachment groups.

3.2. Reattachment mitigates muscle deterioration with tenotomy

Transection of the *soleus* muscle tendon produced a deterioration of *soleus* muscle composition, which was largely mitigated by immediate tendon reattachment (Figs. 2-4). Specific effects of tenotomy in the released muscle, which were fully prevented in reattached muscle, included an increased area percentage of connective tissue (46.5% vs. 16.6%; Fig. 2D), increased percentage of fast type muscle fibers (51.3% compared to 8.9%; Fig. 2C), a reduction in the percentage of slow type muscle fibers (39.6% compared to 77.1%; Fig. 2C), and an increased expression of FAK (1.6 vs. 0.9 units per actin, Fig. 2A) and meta-vinculin expression (23.8 vs. 8.3 units per actin; Fig. 3E).

Re-attachment did mitigate the increase in the percentage of fibers with internal nuclei compared to the tenotomised muscle that was not reattached (32.2% vs. 21.5%; Fig. 2E) but compared to the mock controls from the tenotomised muscle (2.1%) did not prevent it. As well, differences existed between the tenotomised muscle and its mock control for the expression levels of the low abundant FRNK protein, and a 65-kDa and a 55-kDa FAK C-terminus-related protein (Fig. 3C/D), the mass of *soleus* muscle and gamma-vinculin expression levels. The specific phosphorylation of T421/S424-P70S6K and pT183/Y185-JNK differed between the reattached and not reattached muscle for the tenotomised muscle and its mock control (Fig. 2-4). The specific phosphorylation of Y397-FAK was not affected by tenotomy (0.7 ± 0.4 vs. 1.0 ± 0.5, $p = .68$).

3.3. Time course of tenotomy effects

Few, and transient effects were identified in the tenotomised *soleus* muscle until 14 days after Achilles tendon release. These included an increased area percentage of connective tissue and increased percentage of fibers with central cores in tenotomised *soleus* muscle immediately, i.e. 5 min, after tendon release (see Fig. 1 in Ferrié et al., 2019). The percentage of fibers with internal nuclei was increased 4 days after tenotomy in the released muscle (see Fig. 1E in Ferrié et al., 2019). MCSA of muscle fibers in the tenotomised muscle decreases 14 days compared to 4 days after tenotomy (see Fig. 1B in Ferrié et al., 2019). Costamere component expression was not affected 5 min and 4 days after tenotomy (see Fig. 2 in Ferrié et al., data in brief).

MCSA of muscle fibers was transiently increased in mock-tenotomised *soleus* muscle 4 days after tendon release when the specific phosphorylation of pT421/S424-P70S6K was reduced (see Fig. 1B/3B in Ferrié et al., 2019).

3.4. Effects of transfection per se

We characterized the influence of transfection with empty pCMV plasmid on *soleus* muscle compared to not-transfected muscle, and mock controls, in order to later isolate the specific effect of FAK overexpression via transfection with expression plasmid pCMV-FAK (Fig. 5). The mass of the reattached *soleus* muscle, and its mock control, was not affected by transfection with pCMV plasmid respective to the not-transfected muscles (Fig. 6A). Body mass during the two weeks of the experiment increased in the transfected animals, whereas it remained unchanged in the non-transfected animals (Table 1).

Immunohistochemically stained cross-section visualizing the level of FAK (red) in tenotomized *soleus* muscle 14 days after reattachment and transfection with pCMV (A) or pCMV-FAK (B) plasmid. Nuclei appear in blue. Arrows point to fibers demonstrating enhanced FAK staining throughout the sarcoplasma. Bar, 100 µm. Note that the staining intensity was elevated in the interstitium with pCMV transfection. C) Box Whisker plot of the percentage of fibers in pCMV- and pCMV-FAK transfected *soleus* muscle, respectively, demonstrating enhanced FAK staining at the sarcolemma and in the sarcoplasm. +, $p < .05$ vs. pCMV.

Transfection with empty pCMV plasmid altered the composition of

Table 1
Mean ± SD (n) of body mass of the animals in the different groups at the start and end of the three experiments.

Experiment 1				
Tenotomy		5 min	4 days	14 days
Start		256.7 ± 20.3 (6)	250.8 ± 12.3 (6)	257.3 ± 17.2 (6)
	p-value vs 5 min		0.559	0.946
	p-value vs 4 days			0.515
End		271.5 ± 23.5 (6)	257.7 ± 21.4 (6)	276.3 ± 25.5 (6)
	p-value vs 5 min		0.325	0.727
	p-value vs 4 days			0.189
	p-value vs start	0.009	0.297	0.004
Experiment 2				
Tenotomy with reattachment		No_plasmid	pCMV	pCMV-FAK
Start		274.0 ± 11.1 (6)	242.0 ± 7.7 (6)	231.4 ± 10.6 (5)
	p-value vs no_plasmid		< 0.001	< 0.001
	p-value vs pCMV			0.098
End		268.0 ± 12.6 (6)	266.8 ± 6.8 (6)	255.4 ± 10.1 (5)
	p-value vs no_plasmid		0.845	0.059
	p-value vs pCMV			0.083
	p-value vs 14 days tenotomy	0.074	0.490	0.121
Experiment 3				
pCMV-FRNK co-transfection				
Start				184.5 ± 10.6 (6)
End				198.5 ± 11.4 (6)
p-value vs. start				0.001
p-value vs. 14 days tenotomy				< 0.001
p-value vs. reattachment				< 0.001

soleus muscle. Reattachment specific effects of empty transfection concerned the MCSA and fiber type distribution of muscle fibers (Fig. 6B/C), vinculin isoform expression (Fig. 7E/F) and the specific phosphorylation of S2448-mTOR (Fig. 8A): MCSA was 39.5% higher in pCMV-transfected compared to non-transfected mock controls but did not differ in the reattached muscles. The percentage of hybrid type fibers did only differ in the reattached muscles between pCMV-transfected and non-transfected muscles (i.e. 30.5% vs. 14.1%).

Meta- and gamma-vinculin levels and specific phosphorylation of S2448-mTOR in reattached muscles were higher in the pCMV-transfected than non-transfected muscles. The area percentage of connective tissue (increased), the levels of FRNK (decreased; Fig. 7B), and the specific phosphorylation of T183/Y185-JNK (increased) were similarly affected by pCMV-transfection compared to the non-transfected muscle in mock and reattached muscles. Specific phosphorylation of Y397-FAK in mock controls and reattached muscles was reduced in pCMV-transfected muscle compared to non-transfected muscle (Table 2). Mass of the *gastrocnemius medialis* muscles from the legs that were pCMV-transfected were 37% lighter than the non-transfected *gastrocnemii* (0.48 vs 0.76 g, $p = .001$).

3.5. Effects of pCMV-FAK transfection

Transfection with pCMV-FAK plasmid produced muscle fibers, which demonstrated a pronounced increase in the sarcoplasmic staining for FAK (Fig. 5). On average, 8.4 ± 2.4% of muscle fibers in pCMV-FAK transfected muscle demonstrated enhanced FAK staining in the sarcoplasma, whereas only 2.4 ± 0.4% of fibers demonstrated enhanced FAK staining in pCMV-transfected muscle ($p = .01$). FAK staining was also detected at the sarcolemma but the percentage of sarcolemma positive muscle fibers did not differ between pCMV-FAK and pCMV-transfected muscle ($p = .70$). Compared to their mock controls, the specific phosphorylation of Y397-FAK tended to be lower in the pCMV-FAK transfected and reattached muscles and was lower than in pCMV transfected muscle ($p = .094$; Table 2).

Concomitantly, specific effects of re-attachment and pCMV-FAK transfection were identified for the expression of meta-vinculin and gamma-vinculin, and the specific phosphorylation of T183/Y185-JNK and pT421/S424-P70S6K (Fig. 7/8). Vinculin isoform expression was lowered while specific phosphorylation of JNK and P70S6K, was increased, with pCMV-FAK transfection.

The expression of FRNK increased in reattached muscle, and its mock control, with pCMV-FAK transfection compared to pCMV-transfection (Fig. 7B). The level of the 65 kDa protein, p65FAK, being immunoreactive to FAK's C-terminus increased in the reattached muscle compared to its mock control with pCMV-FAK transfection (Fig. 7C). No significant differences were noted for muscle mass, the percentage of fiber types, fibers with internal nuclei and/or central cores and connective tissue in reattached muscles between pCMV-FAK and pCMV-transfection, nor their mock control (Fig. 6).

3.6. Specificity of FAK signaling

Experiments were performed to test the effect of overexpressing the autonomous FAK inhibitor, FRNK, on the phosphorylation status of FAK-modulated signaling molecules in FAK-transfected, not tenotomized *soleus* muscle. Transfection with pCMV-FRNK increased the protein levels of FRNK in pCMV-FAK transfected *soleus* muscle compared to muscle being transfected with pCMV and pCMV-FAK (Fig. 9). FRNK overexpression reduced Y397-phosphorylation of FAK by 23%, respective to FAK overexpression alone. Concomitantly the specific phosphorylation of T183/Y185-JNK was reduced by 30%, and phosphorylation of S63-cJUN was 36% lowered. FRNK and FAK co-overexpression also increased the specific phosphorylation of S2448-mTOR by 8% compared to pCMV-FAK and pCMV- transfected muscle. *Soleus* muscle co-transfected with pCVM-FRNK and pCMV-FAK demonstrated a trend for a 9% lowered mass compared to pCVM- and pCMV-FAK-transfected muscle, i.e. 113.7 ± 19.5 vs. 124.2 ± 11.8 mg ($p = .093$).

3.7. Interrelationships

Over all sample points, the two most connected nodes were p65FAK and FAK with 15 and 13 edges respectively (Fig. 10). The expression levels of FAK correlated positively to those of the costamere-associated

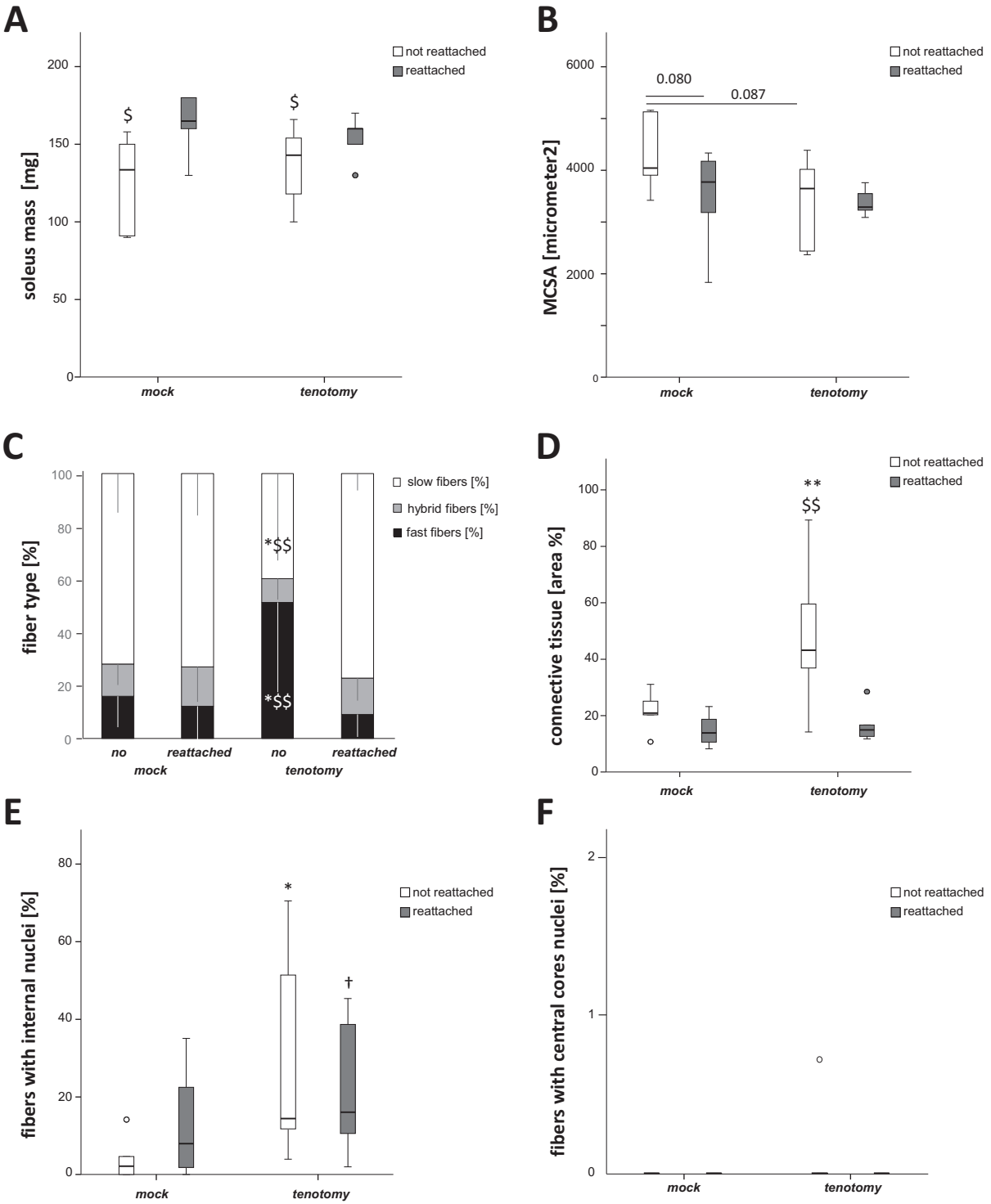

Fig. 2. Soleus muscle composition after Achilles tenotomy and reattachment.
Box Whisker plots of median (line), 95% confidence interval (box) and minima/maxima (whiskers) of anatomical parameters of *soleus* muscles, mass (A), MCSA of muscle fibers (B), connective tissue area percentage (D), percentage of fibers with internal nuclei (E) and fibers with central cores (F), 14 days after tenotomy vs. reattachment, and the respective mock controls. C) mean + standard error of fiber type percentages. Circles indicate outliers. In the case of central core fibers it depicts a single data point with a value above zero. * and **, $p < .05$ and $p < .005$ vs. mock. $ and $$, $p < .05$ and $p < .005$ vs. reattached of the respective tenotomy condition (repeated-measures ANOVA). †, $p < .05$ vs. mock not reattached.

structural proteins (meta- and gamma vinculin) and the specific phosphorylation of S2448-mTOR, pT183/Y185-JNK and T421/S424-P70S6K, as well as the percentages of hybrid type fibers, fibers with internal nuclei and connective tissue, and body mass at the end of the experiment (Fig. 10). The specific phosphorylation of Y397-FAK demonstrated a negative correlation to p65FAK and gamma-vinculin levels. Negative correlations were noted for MCSA to FAK and FRNK expression levels. Transfection with pCMV-FAK, but not pCMV, produced the strongest correlation between the expression levels of FAK and meta-vinculin ($r = 0.817$ vs. 0.419) and produced a positive

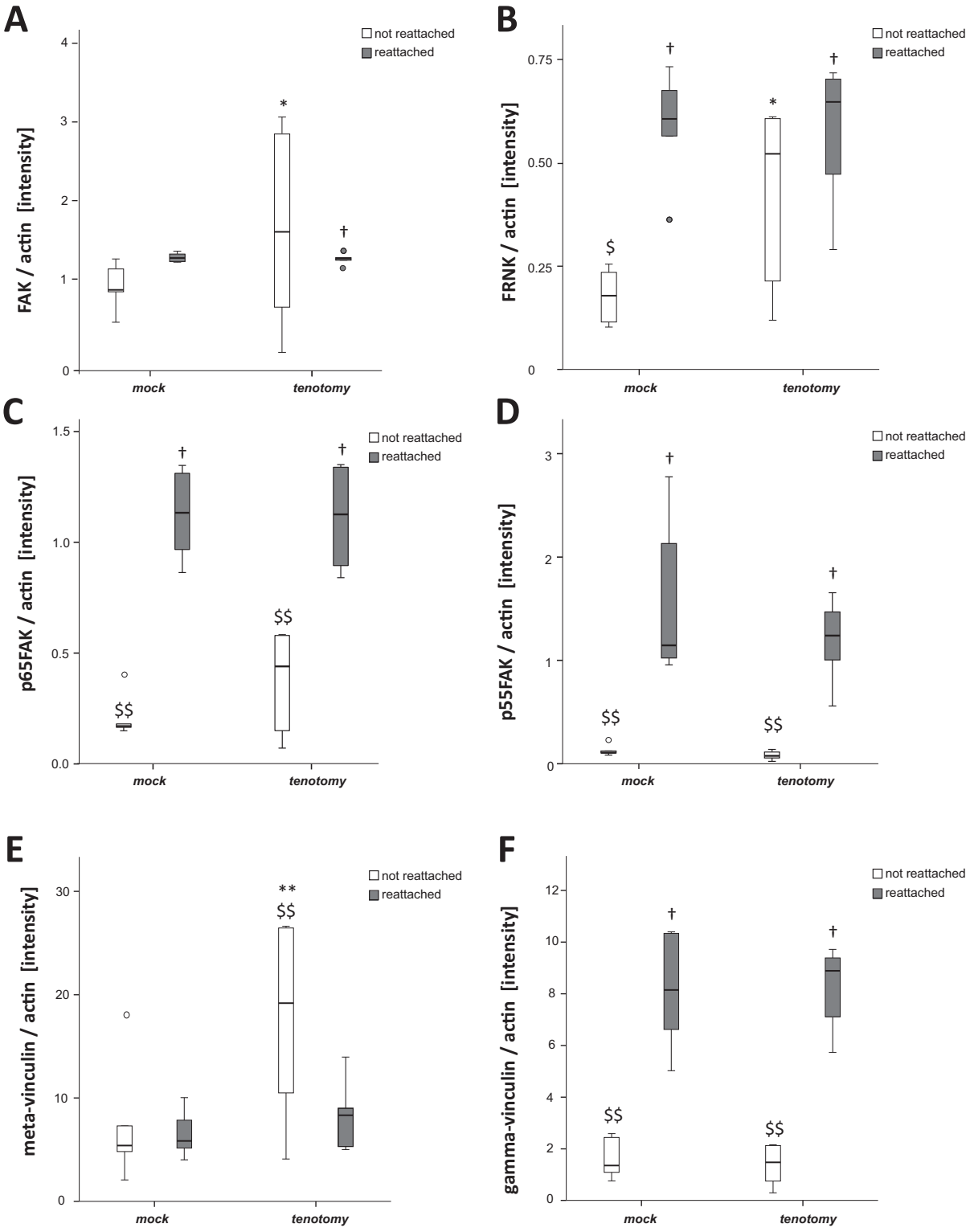

Fig. 3. Costamere component expression after Achilles tenotomy and reattachment.
Box Whisker plots of expression levels for costameric proteins FAK (A), FRNK (B), p65FAK (C), p55FAK (D), meta-vinculin (E), and gamma-vinculin (F) relative to actin, in *soleus* muscles 14 days after tenotomy vs. reattachment, and the respective mock controls. Circles indicate outliers. * and **, $p < .05$ and $p < .005$ vs. mock, $ and $$, $p < .05$ and $p < .005$ vs. reattached of the respective tenotomy condition (repeated-measures ANOVA). †, $p < .05$ vs. mock not reattached.

correlation between meta-vinculin expression and the specific phosphorylation of T183/Y185-JNK.

4. Discussion

Untreated rupture of the Achilles tendon, or late repair (Gross and Nunley, 2017), results in an irreparable functional deficit of the plantar flexor muscle groups due to the irreversible removal of contractile

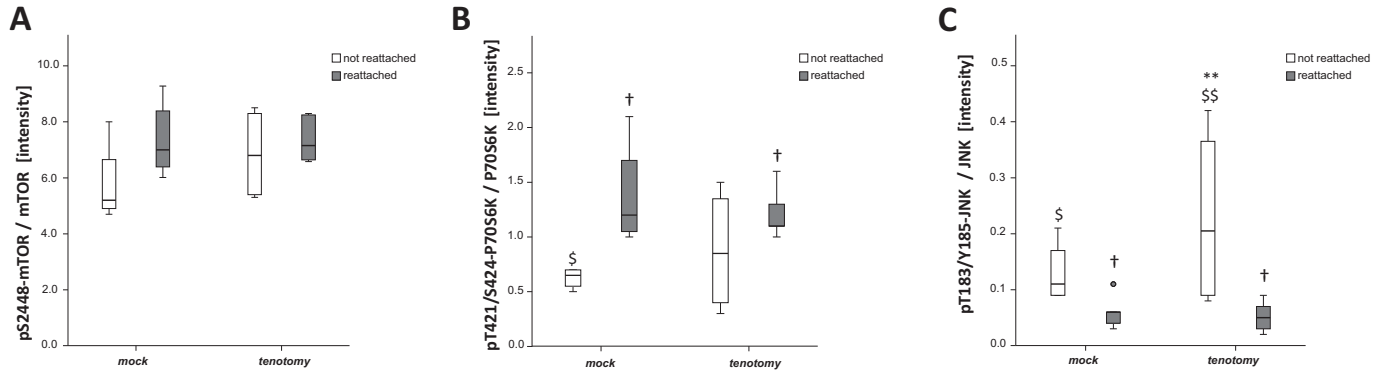

Fig. 4. Hypertrophy signaling after Achilles tenotomy and reattachment.
Box Whisker plots of the specific phosphorylation of mTOR (A), P70S6K (B) and JNK (C) signaling factors in *soleus* muscles 14 days after tenotomy vs. reattachment, and the respective mock controls. Circles indicate outliers, respectively. * and **, $p < .05$ and $p < .005$ vs. mock, $ and $$, $p < .05$ and $p < .005$ vs. reattached (repeated-measures ANOVA). †, $p < .05$ vs. mock not reattached.

elements and the consequent conversion of the muscle into a shorter, fibrous tissue with reduced contractile strength (Hahn et al., 2008; Rahm et al., 2013). There is increasing awareness that the costameric anchoring of myofibrils to the sarcolemma is critical for the stabilization and remodeling of muscle fibers by allowing the functional integration of mechanical forces between contracting muscle fibers, and the subsequent mechano-regulation of gene expression and protein synthesis (Grounds et al., 2005; Durieux et al., 2009; Garcia-Pelagio et al., 2011; Jaka et al., 2015). Knowledge of the temporal course and quantitative implication of changes in costamere- component expression and protein synthetic signaling in skeletal muscle after tendon transection and reconstruction may provide biological guidelines for the optimal window of tendon repair. Specifically it may allow enhancing the functional recovery of the affected muscle-tendon complex by limiting the degree of muscle atrophy through the manipulation of the sarcolemmal anchoring of myofibrils.

Along our hypothesis, our investigation shows specific effects of soleus-targeted Achilles tenotomy on cellular hallmarks of muscle degeneration/regeneration such as fiber type transformation and an increase in the connective tissue (reviewed in (Fluck et al., 2003)), which were mitigated by immediate tendon reattachment. At the molecular level tenotomy-induced alterations, as they were prevented by re-attachment, were reflected by elevated expression of the costamere components FAK and meta-vinculin and specific phosphorylation of T421/S424-JNK in *soleus* muscle. Interestingly, the latter two parameters were further modified by fiber-targeted FAK overexpression upon pCMV-FAK transfection. Along our expectation, certain measured parameters i.e. the percentage of fibers with internal nuclei and FRNK expression levels (Figs. 2/3) which were affected 14 days after tenotomy, had not returned after reattachment to the values in the mock controls of the not-reattached muscles Collectively, the present novel observations highlight the implication of alterations in FAK-regulated costamere composition and signaling in the transformation process of the studied slow tonic *soleus* muscle after tenotomy and indicate that immediate tendon reattachment does not entirely prevent muscle deterioration in the rat.

As reactions to surgical interventions may produce non-specific effects we included controls to identify the robustness and specificity of observed effects. In this regard, we identified that the abundance of FAK C-terminus related proteins and gamma-vinculin (Fig. 3), as well as

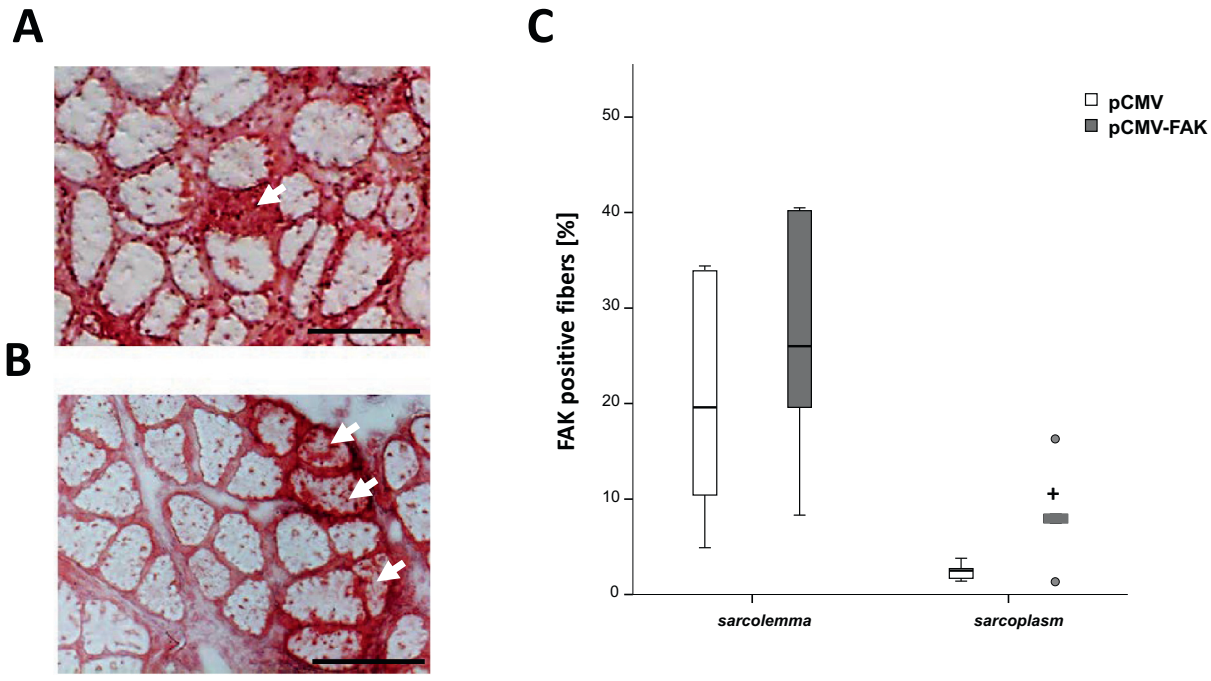

Fig. 5. Effects of transfection in reattached soleus muscle on FAK expression in muscle fibers.

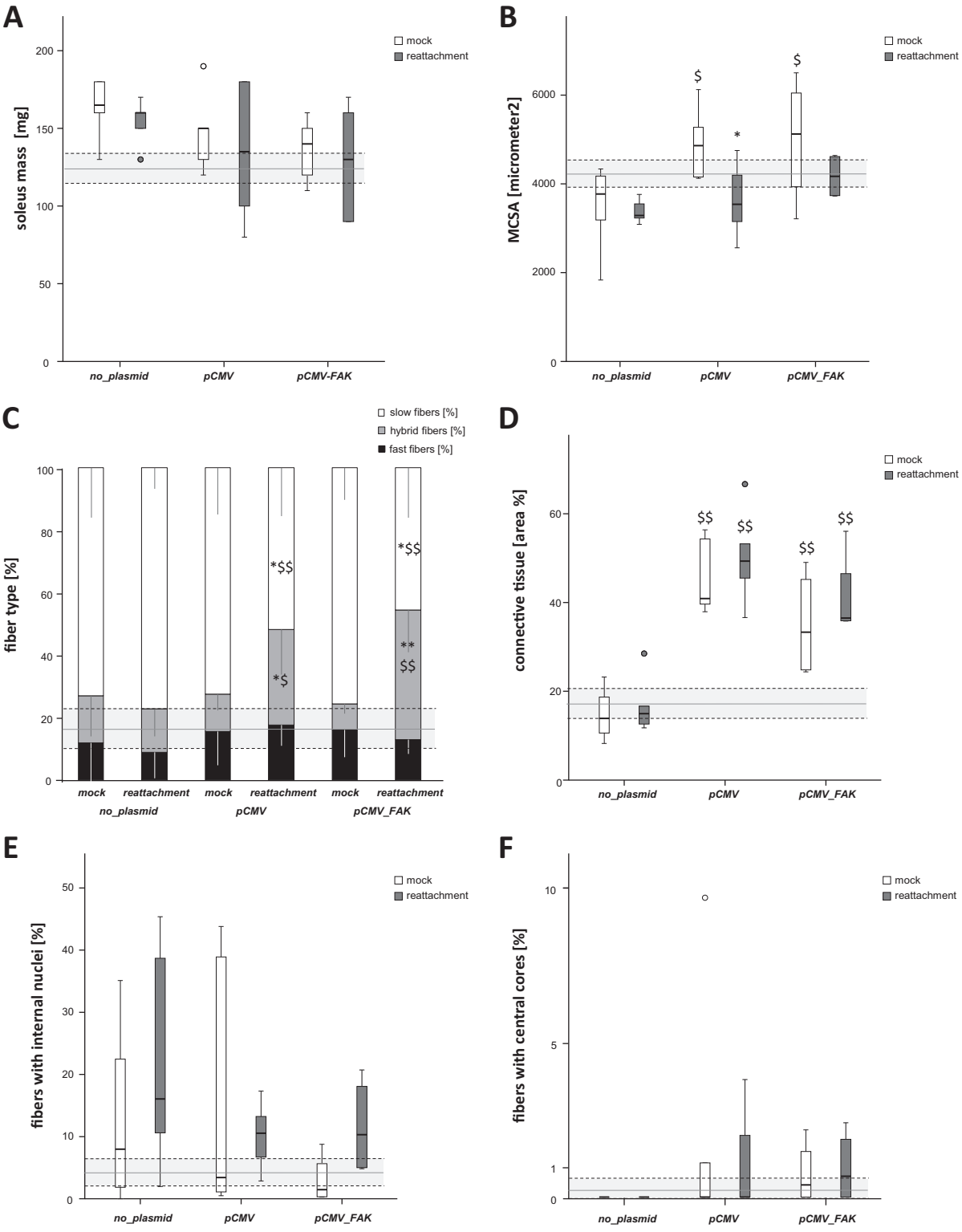

Fig. 6. Effects of transfection on the anatomy of reattached soleus muscle.
Box Whisker plots of anatomical parameters in tenotomized *soleus* muscle, mass (A), MCSA of muscle fibers (B), connective tissue area percentage (D), percentage of fibers with internal nuclei (E) and fibers with central cores (F), 14 days after tenotomy, reattachment, and transfection with the indicated plasmid (no plasmid, pCMV or pCMV-FAK), and the respective mock controls. C) mean + standard error of fiber type percentages (with confidence area for slow type fibers). Circles indicate outliers. * and **, $p < .05$ and $p < .005$ vs. mock; $ and $$, p < .05 and p < .005 vs. no_plasmid; + and + +, p < .05 and p < .005 vs. pCMV (repeated-measures ANOVA). The grey highlighted area denotes the mean ± standard error (confidence area) of the values in the 5 min mock tenotomy control. See Supplementary Fig. S1) for representative examples of the Histological analysis of muscle fiber types.

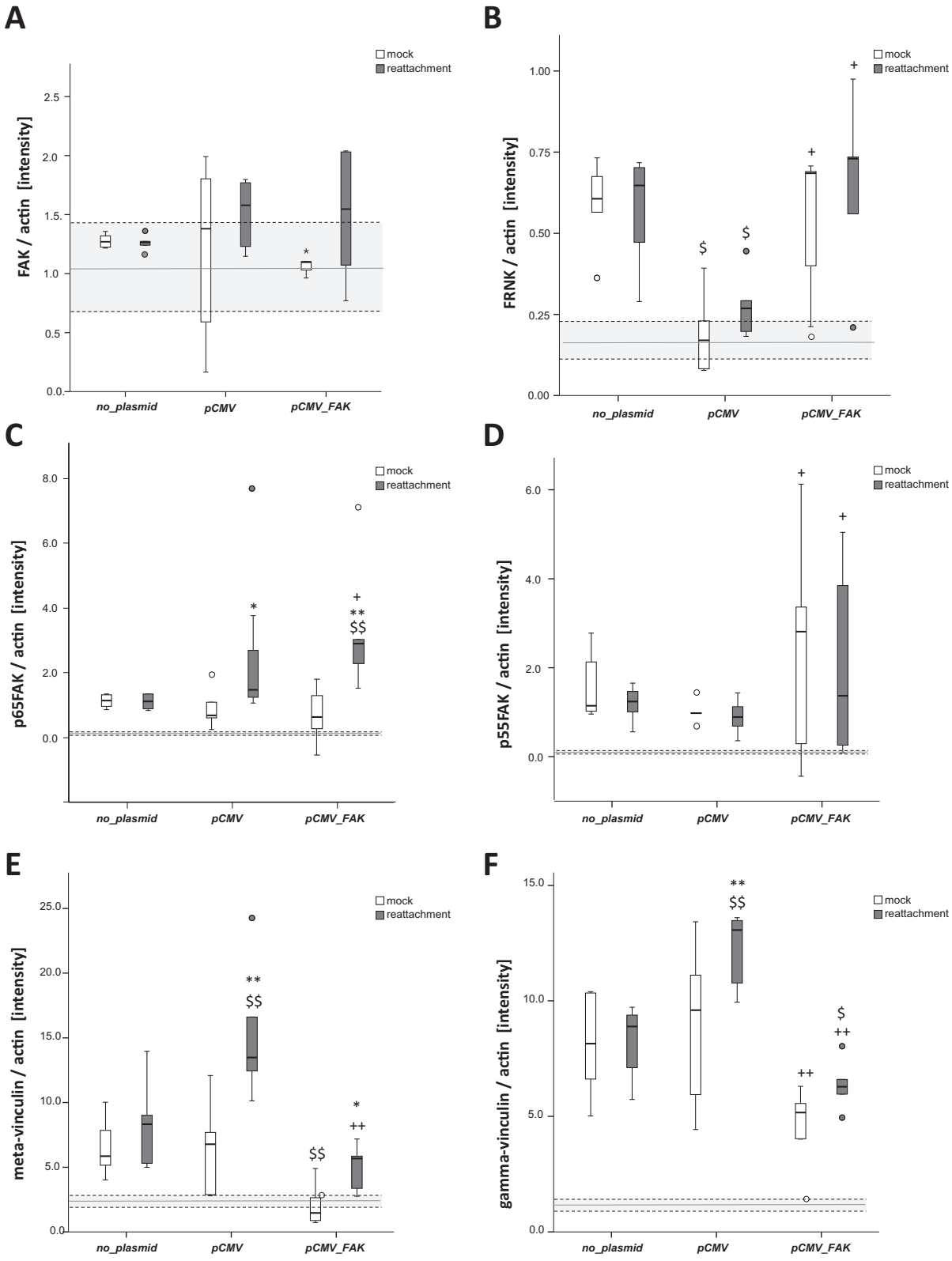

Fig. 7. Effects of transfection on costamere protein expression in reattached soleus muscle.
Box Whisker plots of expression levels for costameric proteins FAK (A), FRNK (B), p65FAK (C), p55FAK (D), meta-vinculin (E), and gamma-vinculin (F), in tenotomized *soleus* muscle 14 days after transfection with the indicated plasmid (no plasmid, pCMV or pCMV-FAK) and reattachment, and the respective mock controls. Circles indicate outliers. * and **, $p < .05$ and $p < .005$ vs. mock; $ and $$, $p < .05$ and $p < .005$ vs. no_plasmid; + and + +, $p < .05$ and $p < .005$ vs. pCMV (repeated-measures ANOVA). The grey highlighted area denotes the mean ± standard error of the values in the (not-transfected) 5 min mock tenotomy control. See Supplementary Fig. S2) for representative immunoblots.

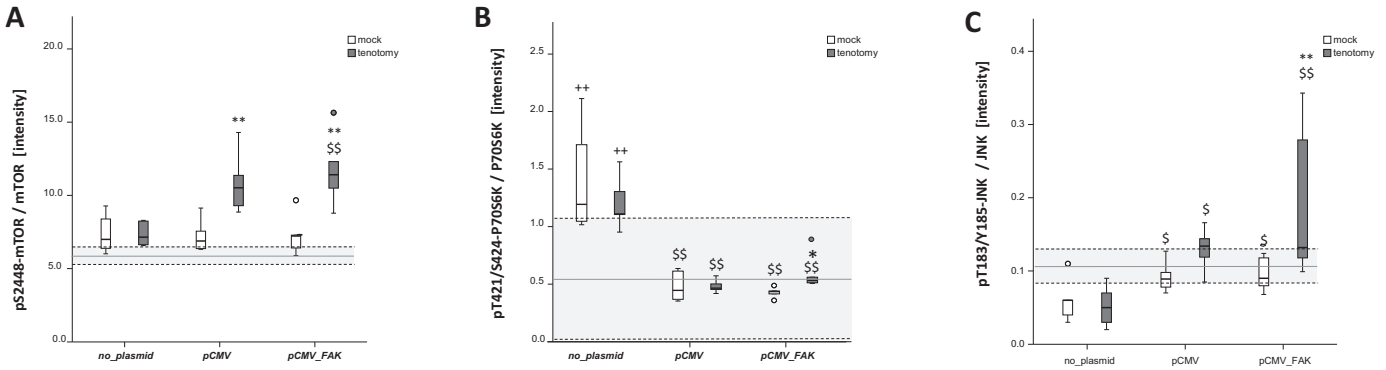

Fig. 8. Effects of transfection on hypertrophy signaling in reattached soleus muscle.
Box Whisker plots of the specific phosphorylation of mTOR (A), P70S6K (B) and JNK hypertrophy signaling factors (C) in tenotomized *soleus* muscle 14 days after transfection with the indicated plasmid (no plasmid, pCMV or pCMV-FAK) and reattachment, and the respective mock controls. Circles indicate outliers. * and **, $p < .05$ and $p < .005$ vs. mock; $ and $$, $p < .05$ and $p < .005$ vs. no_plasmid; + and + +, $p < .05$ and $p < .005$ vs. pCMV (repeated-measures ANOVA). The grey highlighted area denotes the mean ± standard error of the values in the 5 min mock tenotomy control.

the specific phosphorylation of P70S6K and JNK (Fig. 4), differed between the mock controls for the tenotomized and re-attached muscles. In this respect the co-transfection experiments with pCMV-FRNK and pCMV-FAK plasmid, confirmed that FRNK reduces Y397-FAK phosphorylation (Fig. 9; (Klossner, Li et al., 2013)). These experiments also exposed that FRNK overexpression reduced the specific phosphorylation of T183/Y185-JNK and the S63 phosphorylation of the downstream transcription factor cJUN. Collectively, the results imply that the promotion of JNK signaling in *soleus* muscle upon FAK overexpression is related to Y397 phosphorylation of FAK.

A further conspicuous observation was that the fiber MCSA and the specific phosphorylation of T421/S424-P70S6K was affected in the mock tenotomized *soleus* muscle 4 days after tenotomy respective to the baseline control collected 5 min after surgery (see Figs. 1B & 3B in Ferrié et al., 2019). Likely these alterations are due to compensatory muscle reactions in contralateral legs (Lu et al., 1999). Alone an increase was noted for the area percentage of connective tissue and the percentage of fibers with central cores 5 min after tenotomy ((see Fig. 1D/F in Ferrié et al., 2019). Possibly this reflects transient rearrangements in the structural relationships between muscle fiber and connective tissue, and consequent water displacement, in soleus muscle with tenotomy-induced passive muscle shortening. Former mentioned factors possibly explain why slow-to-fast fiber transformation and a trend for a reduced fiber MCSA, appeared as more robust indicators of muscle degeneration than a reduction in the mass of the unrepaired soleus muscle (compare panels A and B/C of Fig. 1 in Ferrié et al., 2019). It follows from the inclusion of the before mentioned mock controls for the different intervention groups, that certain hallmarks of degeneration, such as muscle mass and fibers with central cores, were not specifically modified in soleus muscle by tenotomy but by the surgical manipulation of the tendon (Baker and Hall-Craggs, 1980; Jakubiec-Puka et al., 1992; Abrams et al., 2000; Jamali et al., 2000)) (Fig. 2, Fig. 1 in Ferrié et al., data in brief). Within the limitations of our model, this raises the question whether the surgical exposure of the Achilles tendon, rather than tendon transection, is producing the former affections.

In our study changes in the area percentages of fiber types and the percentage of fibers with internal nuclei, and the costamere components FAK and meta-vinculin, were identified as tenotomy-specific adaptations (Fig. 2C/E, 3A/E). This observation emphasizes active remodeling of fiber composition via fiber type transformation 4–14 days after tendon transection. In contrast to our hypothesis the specific phosphorylation (status) of auto-regulatory sites of the protein synthesis associated signaling factors, mTOR, P70S6K and JNK, in the slow tonic soleus muscle under investigation was not reduced 4 days after tenotomy in the released muscle (see Fig. 3 in Ferrié et al., data in brief). Our expectation was a reduced contractile activity at this time point, which would reduce the basal activation status of these pathways (Gordon et al., 2001; Hornberger et al., 2001). The latter observation possibly reflects the requirement for the enhanced activation of protein synthetic pathways to comfort the elevated need for the synthesis of fast type fiber-associated muscle proteins with tenotomy-induced slow-to-fast transformation of soleus muscle.

Contrary to our expectation, somatic overexpression of FAK concomitant with tendon reattachment did not mitigate cellular aspects of muscle degeneration more than reattachment without transfection. However, FAK overexpression concomitant with tendon reattachment lowered meta-vinculin expression to the levels detected in mock controls immediately after tendon transection that can be considered to reflect the normal situation (Fig. 7C). At the molecular level, the increased specific phosphorylation status of T421/S424-P70S6K and T183/Y185-JNK, after pCMV-FAK transfection also met the expectation that FAK overexpression enhances the drive for protein synthetic signaling (Fig. 8). Along our hypothesis we also observed that FAK expression correlated with the percentage of fibers with internal nuclei, which are reflective of the renewal (i.e. regeneration) of damaged muscle fibers, and slow-to-fast transformation of soleus muscle, which has been found to be affected by FAK expression (Fluck et al., 2002; Fluck et al., 2003). FAK has been shown to be associated with muscle regeneration in correspondence with cytoskeletal alterations (Fluck et al., 1999; Fluck et al., 2003). The effects of pCMV-FAK driven sarcoplasmic FAK overexpression on meta-vinculin levels and the specific phosphorylation of T183/Y185-JNK, and their corresponding correlation with FAK levels, is supportive that FAK is associated with muscle regeneration through a JNK-related cytoskeletal processes. For instance, FAK activation is known to recruit cytoskeletal and signaling

Table 2
Median + SE of specific phosphorylation of Y397-FAK.

	no_plasmid	pCMV	*p*-value vs. no plasmid	pCMV-FAK	no plasmid	p-value vs. pCMV
Mock	0.706 ± 0.268	0.188 ± 0.026	0.061	0.195 ± 0.036	0.049	0.414
Tenotomy	0.996 ± 0.300	0.179 ± 0.032	0.055	0.102 ± 0.008	0.030	0.006
p-value vs. mock	0.508	0.879		0.094		

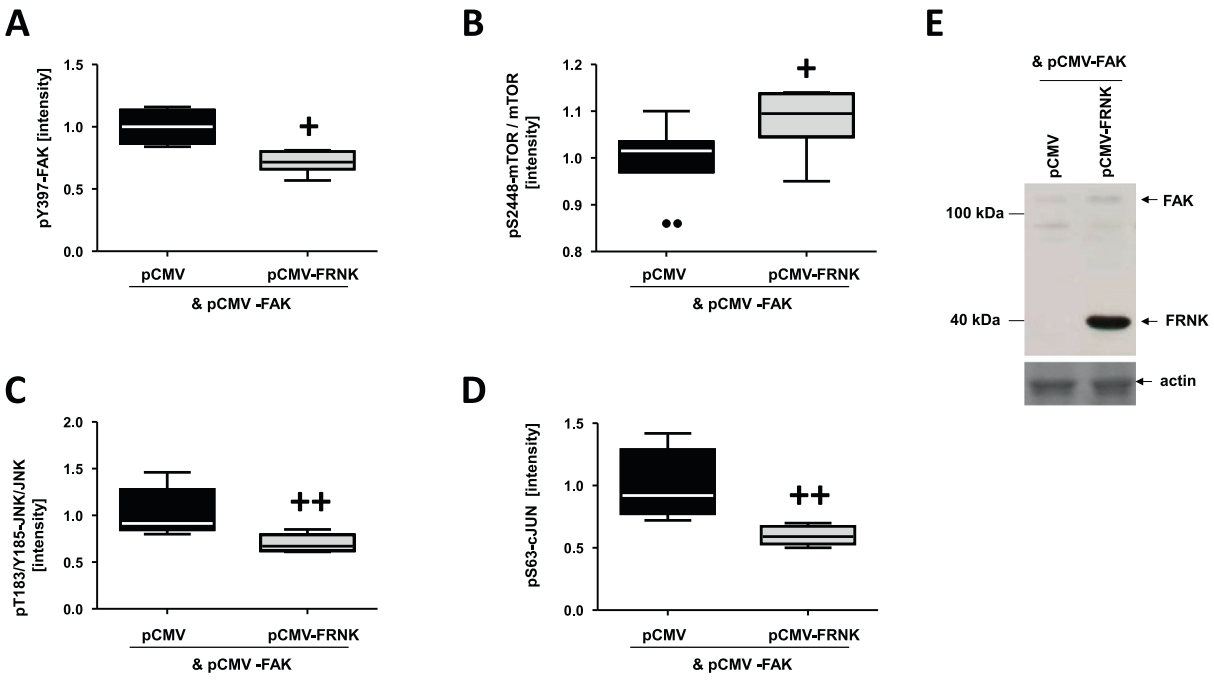

Fig. 9. Effects of FRNK-mediated inhibition of FAK on hypertrophy signaling.
Box Whisker plots of the (specific) phosphorylation of Y397-FAK (A), S2448-mTOR (B), T183/Y185-JNK (C) and S63-cJUN (D) in not tenotomized *soleus* muscle 7 days after co-transfection of muscle pairs with pCMV & pCMV-FAK and pCMV-FRNK & pCMV-FAK. Circles indicate outliers. + and + +, $p < .05$ and $p < .01$ vs. pCMV & pCMV-FAK (repeated-measures ANOVA). Values represented normalized intensities respective to the pCMV & pCMV-FAK transfected muscles. E) Example western blot showing the detection of FAK and FRNK (top) in 10 µg protein homogenate from a *soleus* muscle pair being co-transfected with pCMV & pCMV-FAK and pCMV-FRNK & pCMV-FAK, respectively. Below, respective actin band.

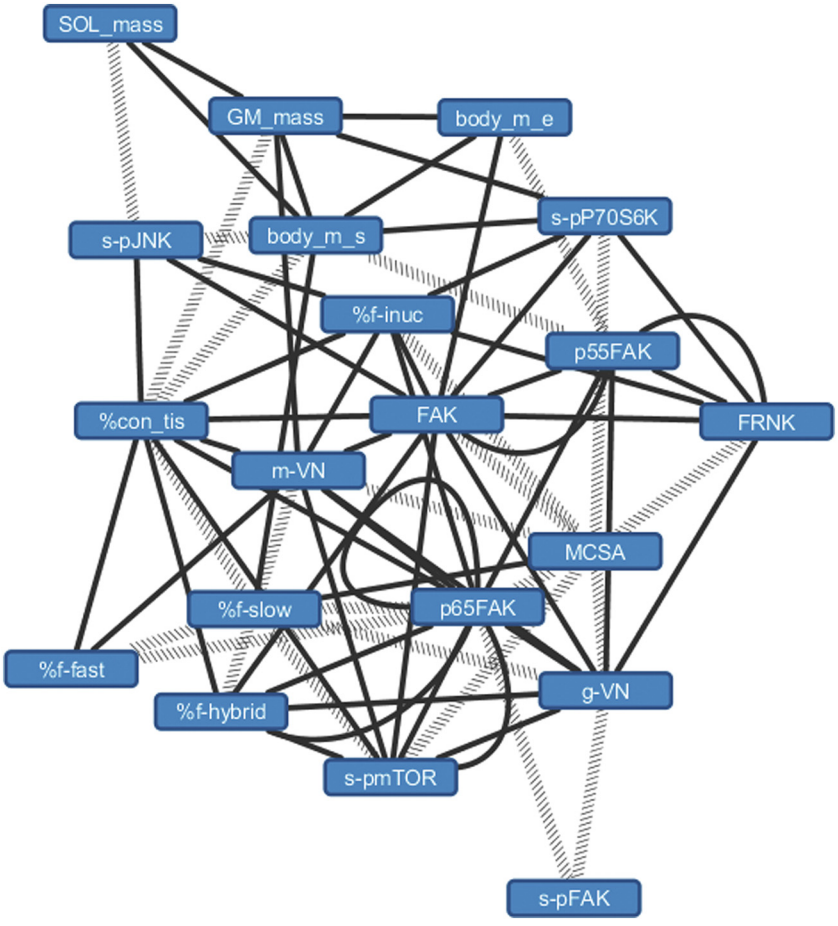

Fig. 10. Inter-relationships.
Hierarchical networks of the correlations between measured molecular and cellular parameters in all assessed *soleus* muscles. Correlated parameters (nodes) are connected though lines (edges; solid dark-grey line: positive correlation, backward slashed grey line: negative correlation). Abbreviations: body_m_e, body mass at the end of the experiment; body_m_s, body mass at the start of the experiment; %con_tis, percentage area of connective tissue; %f-fast, percentage of fast (II) type muscle fibers; %f-hybrid, percentage of hybrid (I/II) type muscle fibers; %f-inuc, percentage of fibers with internal nuclei; %f-slow, percentage of slow (I) type muscle fibers; FAK, levels of FAK; FRNK, levels of FRNK; g-VN, gamma-vinculin; GM_mass, mass of gastrocnemius muscle; MCSA, mean cross sectional area of muscle fibers; m-VN, meta-vinculin; s-pFAK, specific phosphorylation of Y397-FAK; s-pJNK, specific phosphorylation of T183/Y185-JNK; s-pmTOR, specific phosphorylation of S2448-mTOR; s-pP70S6K, specific phosphorylation of T421/S424-P70S6K; SOL_mass, mass of soleus muscle.

factors including JNK to focal adhesions (Giancotti, 1997), and to increase the levels of costamere components in rat soleus muscle (reviewed in (Fluck et al., 1999; Klossner, Li et al., 2013)). The extent to which the effects of FAK overexpression on vinculin isoform expression, JNK phosphorylation and fibers with internal nuclei, may be able to mitigate cellular aspects of fiber degeneration after Achilles tenotomy such as slow-to-fast fiber transformation if administered with another methodology or jointly with other means remains to be further explored.

An interesting aspect of our study was data that show negative correlations between MCSA, FAK and FRNK (Fig. 10). This relationship reflects opposing effects of tenotomy, and re-attachment, on MCSA (i.e. a reduction) and correspondingly increased expression levels of FRNK, and proteins of 55 and 65 kDa in size being related to the FAK C-terminus (i.e. p55FAK and p65FAK (compare Fig. 2B with 3B-D). FRNK regulates sarcomere resting length in cardiomyocytes (Mansour et al., 2004) and it has been found that truncated products from the C-terminus of FAK are produced through the action of the calcium-dependent protease calpain (Carragher et al., 2001; Chan et al., 2010; Zak et al., 2017). This raises the possibility that the relationships between MCSA and FAK C-terminus related proteins reflect a reduction in the resting length of muscle fibers with tendon release, possibly due to increases in the activity of calcium-dependent proteases such as calpain (Ruoss et al., 2018a) subsequent to increases in sarcoplasmic calcium during the observable slow-to-fast fiber transformation (Sreter et al., 1987; Jamali et al., 2000).

Increased levels of FAK C-terminus proteins may also explain the absence of cellular effects of FAK overexpression, as FRNK interferes with the localisation of FAK to focal adhesions and phosphorylation of Y397-FAK in rat soleus muscle (Klossner, Li et al., 2013; Zak et al., 2017); and as we identify that FRNK inhibits JNK signaling (Fig. 9). The inverse relationship between Y397-FAK phosphorylation and the expression of FAK C-terminus related proteins is supported by the negative correlation between the specific phosphorylation of Y397-FAK and the levels of p65FAK ($r = -0.39$), and the increased staining intensity for FAK in the sarcoplasmic compartment with FAK overexpression in re-attached muscle rather than the sarcolemma (Fig. 5). For instance it has been shown that the localisation of FAK to the sarcoplasma, possibly through the binding of FAK to the C-terminal end of myosin heavy chain (Santos et al., 2011), and with the growth of muscle fibers (Durieux et al., 2009). In this respect the opposite effect of FRNK co-overexpression on the specific phosphorylation of S2448-mTOR vis-à-vis T183/Y185-JNK andY397-FAK phosphorylation are intriguing. Antagonistic interactions between mTOR and JNK signaling towards p70S6K have been reported before in rat plantaris muscle after functional overload induced by Achilles tenotomy (Martin et al., 2014). Collectively the observations suggest that enhanced FRNK expression may be part of the hypertrophy-related mechanism allowing gene expression to switch from JNK-cJUN-mediated promotion of muscle transcript expression (Paradis et al., 1996; Andreucci et al., 2002) to the mTOR-mediated activation of protein translation (Zanchi and Lancha Jr., 2008).

A number of limitations may be considered when interpreting our results. Differences in body mass at the start of the experiment between intervention groups and during the intervention (Table 1) indicate that systemic factors presented a potential confounder for differences in certain of anatomical parameters in the studied muscle. This notion is supported by correlations of body mass to soleus mass ($r = 0.250$, $p = .039$) and to FAK levels ($r = 0.275$, $p = .021$). It may also be considered that we did not asses the effect of FRNK overexpression in the situation of tenotomy and reattachment. In this respect, we note that the inhibitory effects of FRNK co-overexpression in 'normal muscle' on phosphorylation of Y397-FAK and T183/Y185-JNK are consistent with an inhibitory role of FAK's C-terminus in the regulation of mechano-transduction down-stream of FAK in muscle cell types (Koshman et al., 2010; Graham et al., 2015). As well we noted that gene transfer introduced a considerable deterioration of the targeted muscle compartment after transfection. This was indicated by an increase in the area percentage of connective tissue of soleus muscle (Fig. 6D), and a 40–50% reduced mass of the respective gastrocnemius medialis muscle ($p = .001$). These findings imply that the observed effects with FAK overexpression in reattached soleus muscle have to be interpreted in reference to an important local disturbance with the deployed transfection method. This includes for instance fibrosis (Eigeldinger-Berthou et al., 2012), which is expected to modify adhesion-related processes such as integrins and coupled intracellular molecules such as FAK (Lagares et al., 2012). Such interferences possibly explain the increased FAK content in the interstitium of soleus muscle after transfection with the empty pCMV plasmid (Fig. 5), and the reduced the levels of FAK in mock controls after pCMV-FAK transfection (Fig. 7A). Possibly the absence of specific cellular effects of the elevated sarcoplasmic content of FAK in pCMV-FAK transfected *soleus* muscle is related to dilution effects, as only ~8% of muscle fibers appeared to overexpress FAK in the sarcoplasm (Fig. 5). Importantly, however, we identify that over both reattached soleus muscle and its mock control, pCMV-FAK transfected muscle demonstrated 9.5% lower values for connective tissue than the pCMV transfected muscle ($p = .031$). For the reattached muscle alone, the difference between the pCMV-FAK and pCMV-transfected muscle amounted to 8.9% but was, similarly as differences in the MCSA of muscle fibers (+554 micormeter2, $p = .161$) not significant ($p = .137$). Last, but not least, despite the respectively important incentives used to control the experiments the degree of muscle activity was not controlled. In this respect the identified 3–5-fold lower specific phosphorylation of Y397-FAK with pCMV- or pCMV-FAK-transfection (Table 2). This parameter, as it is a proxy for mechano-regulation (Rahnert and Burkholder, 2013), is suggestive for an inactivity-related reduction in the activation status of FAK in the studied tonic muscles with tendon reconstruction under transfection.

5. Conclusion

Our findings show that altered expression of the costamere components FAK and meta-vinculin, JNK signaling, as well as slow-to-fast fiber type transformation and an expansion of the connective tissue are involved in the reductive remodeling with tenotomy and specifically prevented by immediate re-attachment. The observations are consistently with the view that enhanced expression of FAK via electro gene transfer, and loading in reattached *soleus* muscle, corrects molecular aspects of reductive fiber remodeling at the level of meta-vinculin expression. However, sarcoplasmic FAK overexpression was insufficient to accelerate a reversal of structural muscle deterioration beyond the effects of reattachment alone. It remains to be tested whether forced remodeling of costameres and downstream hypertrophy signaling via FAK overexpression under a prevented expansion of connective tissue and adjuvant mechanical cues may be able to mitigate muscle degeneration. Lastly, reactions of muscle fibers in mock controls highlight the importance of controlling pathological muscle reactions subsequent to surgical interventions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2019.03.006.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Author contributions

MF: conception and design; FW, CF, SK, MF: method development; CF, SK, FW, MF: performed experiments; CF, SK, FW, MF: analyzed data; SK, MF: results interpretation; SK, MF: figure preparation; CF, MF: drafted the manuscript; CF, SK, MF: editing of manuscript.

Availability of data and material

The data set is publicly available under doi:10.17632/hdmgydpr3v.1 after expiration of the embargo period (14-05-2019).

Acknowledgements

The study was supported by the RESORTHO foundation. We thank Dr. Céline Durieux for assistance during the co-overexpression of FRNK and FAK and Dr. Paola Valdivieso for carrying out immunofluorescent detection of FAK.

References

Abrams, R.A., Tsai, A.M., et al., 2000. Skeletal muscle recovery after tenotomy and 7-day delayed muscle length restoration. Muscle Nerve 23 (5), 707–714.
Andreucci, J.J., Grant, D., et al., 2002. Composition and function of AP-1 transcription complexes during muscle cell differentiation. J. Biol. Chem. 277 (19), 16426–16432.
Aronson, D., Dufresne, S.D., et al., 1997. Contractile activity stimulates the c-Jun NH2-terminal kinase pathway in rat skeletal muscle. J. Biol. Chem. 272 (41), 25636–25640.
Baker, J.H., Hall-Craggs, E.C., 1980. Recovery from central core degeneration of the tenotomized rat soleus muscle. Muscle Nerve 3 (2), 151–159.
Bullard, B., Sainsbury, G., et al., 1990. Digestion of proteins associated with the Z-disc by calpain. J. Muscle Res. Cell Motil. 11 (3), 271–279.
Carragher, N.O., Fincham, V.J., et al., 2001. Cleavage of focal adhesion kinase by different proteases during SRC-regulated transformation and apoptosis. Distinct roles for calpain and caspases. J. Biol. Chem. 276 (6), 4270–4275.
Chan, K.T., Bennin, D.A., et al., 2010. Regulation of adhesion dynamics by calpain-mediated proteolysis of focal adhesion kinase (FAK). J. Biol. Chem. 285 (15), 11418–11426.
Chopard, A., Arrighi, N., et al., 2005. Changes in dysferlin, proteins from dystrophin glycoprotein complex, costameres, and cytoskeleton in human soleus and vastus lateralis muscles after a long-term bedrest with or without exercise. FASEB J. 19 (12), 1722–1724.
Chu, M., Iyengar, R., et al., 2011. Serine-910 phosphorylation of focal adhesion kinase is critical for sarcomere reorganization in cardiomyocyte hypertrophy. Cardiovasc. Res. 92 (3), 409–419.
Dawes, N.J., Cox, V.M., et al., 1996. The induction of c-fos and c-jun in the stretched latissimus dorsi muscle of the rabbit: responses to duration, degree and re-application of the stretch stimulus. Exp. Physiol. 81 (3), 329–339.
Durieux, A.C., D'Antona, G., et al., 2009. Focal adhesion kinase is a load-dependent governor of the slow contractile and oxidative muscle phenotype. J. Physiol. 587 (Pt 14), 3703–3717.
Egger, A.C., Berkowitz, M.J., 2017. Achilles tendon injuries. Curr. Rev. Musculoskelet. Med. 10 (1), 72–80.
Eigeldinger-Berthou, S., Buntschu, P., et al., 2012. Electric pulses augment reporter gene expression in the beating heart. J. Gene Med. 14 (3), 191–203.
Ferrié, C., Kasper, S., Wanivenhaus, F., Flück, M., 2019. Time Course of Costamere-Related Alterations in Focal Adhesion Signaling and Composition of Rat Soleus Muscle after Achilles Tenotomy, Data in Brief, Submitted.
Fluck, M., Carson, J.A., et al., 1999. Focal adhesion proteins FAK and paxillin increase in hypertrophied skeletal muscle. Am. J. Phys. 277 (1 Pt 1), C152–C162.
Fluck, M., Ziemiecki, A., et al., 2002. Fibre-type specific concentration of focal adhesion kinase at the sarcolemma: influence of fibre innervation and regeneration. J. Exp. Biol. 205 (Pt 16), 2337–2348.
Fluck, M., Chiquet, M., et al., 2003. "Reloading of atrophied rat soleus muscle induces tenascin-C expression around damaged muscle fibers." American journal of physiology. Regul. Integr. Comp. Physiol. 284 (3), R792–R801.
Fluck, M., Li, R., et al., 2014. Early changes in costameric and mitochondrial protein expression with unloading are muscle specific. Biomed. Res. Int. 2014, 519310.
Franchi, M.V., Ruoss, S., et al., 2018. Regional regulation of focal adhesion kinase after concentric and eccentric loading is related to remodeling of human skeletal muscle. Acta Physiol. 223 (3) e13056.
Fujii, N., Boppart, M.D., et al., 2004. Overexpression or ablation of JNK in skeletal muscle has no effect on glycogen synthase activity. Am. J. Phys. Cell Physiol. 287 (1), C200–C208.
Garcia-Pelagio, K.P., Bloch, R.J., et al., 2011. Biomechanics of the sarcolemma and costameres in single skeletal muscle fibers from normal and dystrophin-null mice. J. Muscle Res. Cell Motil. 31 (5–6), 323–336.
Giancotti, F.G., 1997. Integrin signaling: specificity and control of cell survival and cell cycle progression. Curr. Opin. Cell Biol. 9 (5), 691–700.
Goll, D.E., Thompson, V.F., et al., 2003. The calpain system. Physiol. Rev. 83 (3), 731–801.
Goll, D.E., Neti, G., et al., 2008. Myofibrillar protein turnover: the proteasome and the calpains. J. Anim. Sci. 86 (14 Suppl), E19–E35.
Gordon, S.E., Fluck, M., et al., 2001. Selected contribution: skeletal muscle focal adhesion kinase, paxillin, and serum response factor are loading dependent. J. Appl. Physiol. 90 (3), 1174–1183 (discussion 1165).
Graham, Z.A., Gallagher, P.M., et al., 2015. Focal adhesion kinase and its role in skeletal muscle. J. Muscle Res. Cell Motil. 36 (4–5), 305–315.
Gross, C.E., Nunley, J.A., 2017. Treatment of Neglected Achilles Tendon Ruptures with Interpositional Allograft. Foot Ankle Clin. 22 (4), 735–743.
Grounds, M.D., Sorokin, L., et al., 2005. Strength at the extracellular matrix-muscle interface. Scand. J. Med. Sci. Sports 15 (6), 381–391.
Hahn, F., Meyer, P., et al., 2008. Treatment of chronic achilles tendinopathy and ruptures with flexor hallucis tendon transfer: clinical outcome and MRI findings. Foot Ankle Int. 29 (8), 794–802.
Herdegen, T., Claret, F.X., et al., 1998. Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. J. Neurosci. 18 (14), 5124–5135.
Hornberger, T.A., Hunter, R.B., et al., 2001. Regulation of translation factors during hindlimb unloading and denervation of skeletal muscle in rats. Am. J. Phys. Cell Physiol. 281 (1), C179–C187.
Hoshijima, M., 2006. "Mechanical stress-strain sensors embedded in cardiac cytoskeleton: Z disk, titin, and associated structures." American journal of physiology. Heart Circ. Physiol. 290 (4), H1313–H1325.
Jaka, O., Casas-Fraile, L., et al., 2015. Costamere proteins and their involvement in myopathic processes. Expert Rev. Mol. Med. 17, e12.
Jakubiec-Puka, A., Catani, C., et al., 1992. Myosin heavy-chain composition in striated muscle after tenotomy. Biochem. J. 282 (Pt 1), 237–242.
Jamali, A.A., Afshar, P., et al., 2000. Skeletal muscle response to tenotomy. Muscle Nerve 23 (6), 851–862.
Klossner, S., Durieux, A.C., et al., 2009. Mechano-transduction to muscle protein synthesis is modulated by FAK. Eur. J. Appl. Physiol. 106 (3), 389–398.
Klossner, S., Li, R., et al., 2013. "Quantitative changes in focal adhesion kinase and its inhibitor, FRNK, drive load-dependent expression of costamere components." American journal of physiology. Regul. Integr. Comp. Physiol. 305 (6), R647–R657.
Koshman, Y.E., Kim, T., et al., 2010. FRNK inhibition of focal adhesion kinase-dependent signaling and migration in vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 30 (11), 2226–2233.
Lagares, D., Busnadiego, O., et al., 2012. Inhibition of focal adhesion kinase prevents experimental lung fibrosis and myofibroblast formation. Arthritis Rheum. 64 (5), 1653–1664.
Li, R., Narici, M.V., et al., 2013. Costamere remodeling with muscle loading and unloading in healthy young men. J. Anat. 223 (5), 525–536.
Lu, D.X., L, K.A., et al., 1999. Experimental changes to limb muscles elicit contralateral reactions: the problem of controls. J. Exp. Biol. 202 (Pt 12), 1691–1700.
Mansour, H., de Tombe, P.P., et al., 2004. Restoration of resting sarcomere length after uniaxial static strain is regulated by protein kinase Cepsilon and focal adhesion kinase. Circ. Res. 94 (5), 642–649.
Martin, T.D., Dennis, M.D., et al., 2014. mTORC1 and JNK coordinate phosphorylation of the p70S6K1 autoinhibitory domain in skeletal muscle following functional overloading. Am. J. Physiol. Endocrinol. Metab. 306 (12), E1397–E1405.
Mayhew, T.M., 1991. The new stereological methods for interpreting functional morphology from slices of cells and organs. Exp. Physiol. 76 (5), 639–665.
McNeil, P.L., Kirchhausen, T., 2005. An emergency response team for membrane repair Nature reviews. Mol. Cell. Biol. 6 (6), 499–505.
Midwood, K.S., Schwarzbauer, J.E., 2002. Tenascin-C modulates matrix contraction via focal adhesion kinase- and Rho-mediated signaling pathways. Mol. Biol. Cell 13 (10), 3601–3613.
Narici, M.V., Flueck, M., et al., 2011. Skeletal muscle remodeling in response to alpine skiing training in older individuals. Scand. J. Med. Sci. Sports 21 (Suppl. 1), 23–28.
Oktay, M., Wary, K.K., et al., 1999. Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. J. Cell Biol. 145 (7), 1461–1469.
Paradis, P., MacLellan, W.R., et al., 1996. Serum response factor mediates AP-1-dependent induction of the skeletal alpha-actin promoter in ventricular myocytes. J. Biol. Chem. 271 (18), 10827–10833.
Pardo, J.V., Siliciano, J.D., et al., 1983. A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. Proc. Natl. Acad. Sci. U. S. A. 80 (4), 1008–1012.
Peter, A.K., Cheng, H., et al., 2011. The costamere bridges sarcomeres to the sarcolemma in striated muscle. Prog. Pediatr. Cardiol. 31 (2), 83–88.
Rahm, S., Spross, C., et al., 2013. Operative treatment of chronic irreparable Achilles tendon ruptures with large flexor hallucis longus tendon transfers. Foot Ankle Int. 34 (8), 1100–1110.
Rahnert, J.A., Burkholder, T.J., 2013. High-frequency electrical stimulation reveals a p38-mTOR signaling module correlated with force-time integral. J. Exp. Biol. 216 (Pt 14), 2619–2631.
Ramanujam, C.L., Zgonis, T., 2017. Surgical correction of the Achilles tendon for diabetic foot ulcerations and charcot neuroarthropathy. Clin. Podiatr. Med. Surg. 34 (2), 275–280.
Ruoss, S., Kindt, P., et al., 2018a. Inhibition of calpain delays early muscle atrophy after rotator cuff tendon release in sheep. Phys. Rep. 6 (21), e13833.
Ruoss, S., Mohl, C.B., et al., 2018b. Costamere protein expression and tissue composition of rotator cuff muscle after tendon release in sheep. J. Orthop. Res. 36 (1), 227–281.
Santos, A.M., Schechtman, D., et al., 2011. FERM domain interaction with myosin negatively regulates FAK in cardiomyocyte hypertrophy. Nat. Chem. Biol. 8 (1), 102–110.
Sharp, W.W., Simpson, D.G., et al., 1997. Mechanical forces regulate focal adhesion and costamere assembly in cardiac myocytes. Am. J. Phys. 273 (Pt 2), H546–H556.
Sparrow, J.C., Schock, F., 2009. The initial steps of myofibril assembly: integrins pave the way nature reviews. Mol. Cell. Biol. 10 (4), 293–298.
Sreter, F.A., Lopez, J.R., et al., 1987. Changes in intracellular ionized Ca concentration associated with muscle fiber type transformation. Am. J. Phys. 253 (2 Pt 1), C296–C300.
Sunderland, S., Lavarack, J.O., 1959. Changes in human muscles after permanent tenotomy. J. Neurol. Neurosurg. Psychiatry 22, 167–174.
Zak, T.J., Koshman, Y.E., et al., 2017. Regulation of Focal Adhesion Kinase through a Direct Interaction with an Endogenous Inhibitor. Biochemistry 56 (35), 4722–4731.
Zanchi, N.E., Lancha Jr., A.H., 2008. Mechanical stimuli of skeletal muscle: implications on mTOR/p70s6k and protein synthesis. Eur. J. Appl. Physiol. 102 (3), 253–263.